GRADUATE TRAINING PROGRAM IN BASIC RESEARCH EMPLOYING BIOTECHNOLOGY

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The Army Research Office Graduate Training Program in Basic Research Employing Biotechnology at Northwestern University supported three students for a period of one year each from July 1, 1986 through June 30, 1989. This report summarizes the research activities of the three students during the period of support.

Stephanie S. Watowich's studies focused on genes controlling the synthesis of heat shock proteins. All eukaryotic cells, including those from humans, respond to a variety of environmental stresses by increasing the synthesis of a family of stress-induced or heat shock proteins. The goal of Ms. Watowich's research was to identify and characterize other genes and proteins in the human cell which share features with the heat shock proteins. In particular, she identified human genomic clones which encode distinct proteins related to human HSP70, including a glucose-related protein and a protein involved in the removal of clathrin cages from intracellular vesicles. Her results suggest a complex pattern of gene regulation which in dependent on the specificity and magnitude of physiological damage to the cell.

Wendy L. Niebling's research relates to the function of the immune system, and involved the mechanisms by which antigens stimulate resting B lymphocytes to proliferate and differentiate into antibody-secreting cells. Specifically, she studied the cycling pathways of B cell surface receptors to determine possible means of delivering antigen into the processing pathway more efficiently. Her research may lead to the development of efficient ways for delivering antigens which can block autoimmune responses.

Kevin P. Foley's research was aimed at understanding how genes become activated in response to intrinsic and extrinsic stimuli, and focused on characterizing the trans-acting factors which interact with regulatory elements of the β-globin gene during red blood cell development. He has used both genetic and immunochemical methods to study a particular protein, βE-P5, that is involved in the termination of transcription. His research will have important implications for understanding the mechanisms underlying developmental control of gene regulation.

SUMMARY OF RESEARCH

A. Stephanie S. Watowich

All eukaryotic cells, including those from humans, respond to heat shock and a variety of other environmental stresses by the increased synthesis of a small family of heat shock or stress-induced proteins. The major heat inducible protein in eukaryotic cells, HSP70, is antigenically related to other cross-hybridizing sequences in the human genome sharing homology with the HSP70 gene. Some of the members of the 70kd protein family have recently been studied in other laboratories and shown to be similar to or identical with a glucose-related protein as well as a bovine brain enzyme, the clathrin uncoating ATPase.

The goal of the research of Stephanie Watowich is to identify and characterize other genes and proteins in human cells which share features with HSP70. She has identified human genomic DNA clones containing sequences which encode distinct proteins related to HSP70, including a glucose-rated protein, GRP74, and a protein involved in removing clathrin cages from vesicles, known as the uncoating ATPase. Partial sequence analysis of the GRP74 clone reveals extensive hemology (70%) with human HSP70 as well as with a glucose-responsive gene isolated from rat. Genomic analysis has demonstrated that at least two copies of GRP74-related sequences are present in human cells.

Incubation of human (HeLa) cells with 2-deoxyglucose, an inhibitor of protein glycosylation, induces GRP74 mRNA approximately 5-10 fold above control levels following 8-12 hours of incubation. Concomitant with an increase in GRP74 mRNA levels is the induced synthesis of the GRP74 protein. During 2-deoxyglucose treatment, the level of GRP74 increases the level of constitutively synthesized HSP70 decreases. Upon recovery from 2-deoxyglucose treatment, the synthesis of GRP74 mRNA and protein decreases while the synthesis of HSP70 mRNA and protein is transiently induced above control levels. Future experiments will be designed to study whether the expression of HSP70 and GRP74 are inversely related, and to determine the level at which this stress response is controlled.

Under normal conditions of cell growth, HeLa cells express three proteins related to the major heat shock protein HSP70: a glucose responsive protein, GRP78; the clathrin uncoating ATPase, P72; and HSP70. Stephanie has isolated human genomic sequences which encode GRP78. She has used these sequences along with the cloned HSP70 gene to study the expression of GRP78 and HSP70 in response to specific and general inhibitors of glycoprotein processing. These induce GRP78 synthesis and mRNA accumulation. A second class of chemicals stimulates the expression of GRP78 and HSP70; this class includes agents which affect protein conformation and structure such as amino acid analogues and heavy metals. The third class of chemicals, which include the calcium ionophore A23187 and the glucose analogue 2-deoxyglucose, are agents which have been used in previous studies on GRP78 expression and which exert pleitropic effects upon the cell. The increase in GRP78 expression is due to transcriptional activation whereas the repression of HSP70 is regulated post-transcriptionally.

These results suggest a complex pattern of regulation for the GRP78 and HSP70 genes which is dependent on the specificity and magnitude of physiological damage sustained by the cell.

The goal of Stephanie's current research is to understand the molecular events which activate the expression of the glucose-regulated protein, GRP78, during paramyxovirus (SV5) infection. The GRP78 protein associates with the viral glycoprotein HN during SV5 infection. Transcription of GRP78 is activated 9-12 hours post-infection. This activation occurs several hours after viral protein synthesis begins and HN GRP78 complexes are detected. Her hypothesis is that the activation of GRP78 transcription occurs in response to the rapid rate of viral glycoprotein (HN) synthesis during infection. She is testing this hypothesis by constructing inducible expression vectors which are capable of directing the synthesis of individual viral proteins. By studying the relationship between the synthesis rate of the viral protein (HN), the extent of interaction between the viral protein and GRP78, and the transcription rate of GRP78 she hopes to understand the mechanism which activates GRP78 transcription during the viral infection.

B. Wendy L. Niebling

Wendy L. Niebling's research is concerned with the function of the immune system and focused on characterizing the cycling pathways of B cell surface receptors. Her objective is to determine possible means of delivering antigen into the antigen-processing pathway more efficiently. By covalently coupling antigen to antibodies directed against different B cell surface receptors, and subsequently measuring the ability of these B cells to present antigen and activate T cells, she will be able to determine which recycling pathways intersect the processing pathway.

The means by which the antigenic peptide is held on a fixed B cell surface in a stimulatory fashion is not understood. Using the same approach, that is, by coupling the peptide to immunoglobulin, this cell surface phenomenon may be explored. Determining whether the activating ability of peptide-antibody conjugates can be blocked by a mouse peptide which blocks peptide pulsing, may lead to the development of efficient ways to deliver antigens which block autoimmune responses.

The advantages of using the antigen-antibody conjugate *in vivo* have not been fully explored. These advantages may include efficient delivery of antigen to the specific cells involved in the immune response, namely B cells and possibly macrophages, and a means of delivering these antigens in a non-evasive fashion. The peptide-antibody conjugates may therefore be able to prime a T cell response *in vivo*.

Currently Wendy is purifying the antibodies for use in these studies. These antibodies include anti-transferrin receptor, anti-Fc receptor, rabbit anti-mouse (Fab)'2, and anti-MHC molecules. She is also working with the synthetic mouse cytochrome c peptide, Cys92-104, to determine if a peptide-antibody conjugate will block the response to a native cytochrome c protein.

Helper T cells require the processing of globular proteins to produce a peptide fragment which contains the T cell antigenic determinant. The C-terminal peptide fragment of pigeon cytochrome c (Pc81-104) does not require

processing in order to be presented to an antigen-specific T cell hybrid (TPc9.1-3). A time course of peptide presentation reveals that splenic B cells, serving as antigen presenting cells, maximally present peptide after 2 hours in culture and lose their ability to present Pc81-104 after 24 hours of continuous peptide pulsing. Wendy has been investigating this loss of presenting ability by peptide-pulsed B cells. The loss of presenting ability can be correlated with the antigen's MHC restriction, as B cells pulsed with Pc81-104 (I-EK restricted) are capable of presenting OVA (I-AK restricted) but are impaired in their ability to present another I-EK restricted peptide (HSV1-23).

In addition to investigating the presenting ability of pulsed B cells, she has also been examining supernatants from these cells for possible antigenic complexes formed by peptide interaction with serum albumin contained in the culture medium. The verification and kinetics of albumin as a peptide binding protein are currently being further characterized.

C. Kevin P. Foley

Kevin Foley's research involved characterizing the trans-acting factors which interact with regulatory elements of the chicken adult β-globin gene. These factors govern both tissue and developmental stage-specific regulation of β-globin by binding to specific sites in the enhancer and promoter region of the gene. Kevin has been analyzing a protein which binds at the 3' end of the enhancer. This work has been approached both genetically and immunochemically.

The starting point for the genetic analysis of βE-P5 has been the hypothesis that this protein is involved in the termination of transcription. This hypothesis was based on observations (1) that the binding site is located 3' to the gene, (2) that homologous sequences have been found 3' to other genes and have been implicated in transcript stability, and (3) that the enhancer does not function if it is relocated 5' to the promoter.

In order to test this, Kevin constructed deletion mutants of the enhancer in a β-globin expression vector. He analyzed their effects on transcription in transient transfection assays, using an AEV-transformed RBC-precursor cell line. The results of S1-nuclease protection analysis with isolated mRNA indicated that deletion of the βE-P5 binding site alone was not sufficient to decrease the stability of the transcript. Further analysis is now underway to determine why the enhancer must be located 3' to the gene, and whether any other of the adjacent binding sites are involved in transcript stability or termination.

The approach he has used in the immunochemical analysis of βE-P5 has been similar to an approach used in his laboratory for other promoter and enhancer-binding factors: (1) affinity column purification of βE-P5 from red blood cell extracts, using DNA oligonucleotides (containing the binding site of interest) coupled to the column matrix; (ii) screening the culture supernatants of hybridomas derived from mice immunized with crude and purified extracts (by a highly sensitive "reverse" RIA procedure developed in his laboratories for anti-βE-P5 monoclonal antibodies); (iii) confirming the binding site of the proteins bound by these antibodies using a footprint immunoselection procedure he and his colleagues developed; (iv) identifying the protein using the techniques western blotting on crude and purified extracts; and (v) screening a λgt11 expression library for cDNA clones which encode the protein.

Kevin is analyzing a number of putative anti-βE-P5 antibodies which were identified by the reverse RIA procedure described above. Two early candidates have now been shown to be antibodies against a factor which binds the β-globin promoter and is highly important in the developmental stage-specific expression of the gene; further characterization and cDNA isolation are now being pursued.

Kevin has continued to characterize the trans-acting factors which interest with the chicken adult β-globin gene promoter and enhancer elements. Through the use of the footprint immunoselection and the reverse RIA techniques, two monoclonal antibodies have been isolated which recognize a 65 kDa protein. This protein has been shown to bind two similar sites within the promoter and enhancer of the β-globin gene. By Western blot analysis of extracts prepared from various erythroid and non-erythroid cell types, this factor has been found to be expressed in a stage and tissue-specific manner in mature adult erythrocytes. DNase 1 footprinting analysis has also shown that only extracts of cells which express adult β-globin are capable of footprinting the promoter binding site for NF-E3. In addition, this footprint is present in mature adult erythrocytes but absent in mature embryonic erythrocytes. Since embryonic erythropoiesis in chickens is associated with a switch from the expression of embryonic ∈-globin to the adult β-globin isotype, he proposed that NF-E3 regulates this switch by activating transcription of the adult β-globin gene.

Unlike the promoter site, the enhancer binding site for NF-E3, is footprinted in all types of extracts examined. During biochemical purification of NF-E3, using promoter and enhancer site-specific DNA affinity columns, another ubiquitous factor copurifies with NF-E3. This factor, βP-P4/βE-P3, footprints two non-NF-E3 sites in the β-promoter and enhancer. Therefore, it appears that the NF-E3 enhancer binding site is footprinted by βP-P4/βE-P3 in extracts which do not contain NF-E3. As might be expected, the banding pattern of the footprint in the absence of NF-E3 is distinct from that obtained when the extracts contain NF-E3. It is not clear whether this observed interaction occurs *in vivo*.

Kevin's goal is to examine *in vivo* the role of NF-E3 in the transcriptional activation of the adult β-globin gene in erythrocytes, and in the concomitant suppression of the ∈-globin gene. A necessary first step is the isolation of a cDNA clone encoding the factor. He is pursuing this by screening several erythroid λgt11 expression libraries with the two anti-NF-E3 monoclonal antibodies. Several putative clones have been isolated and he is currently characterizing these clones to determine whether they encode the NF-E3 factor.

List of manuscripts submitted or published under ARO sponsorship, including journal references:

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